# Kinetic and thermodynamic studies of peptidyltransferase in ribosomes from the extreme thermophile *Thermus thermophilus*

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**ABSTRACT**

**Throughout evolution, emerging organisms survived by adapting existing biochemical processes to new reaction conditions. Simple protein enzymes balanced changes in structural stability with changes that permitted optimal catalysis by adjustments in both entropic and enthalpic contributions to the free energy of activation for the reaction. Study of adaptive mechanisms by large multicomponent enzymes such as the ribosome has been largely unexplored. Here we have determined the kinetic and thermodynamic parameters of peptidyltransferase in ribosomes from the extreme thermophile *Thermus thermophilus*. Activity of thermophilic enzymes can be assayed over a wide range of temperatures, enabling one to measure accurate catalytic rates and determine enthalpic and entropic contributions to the free energy of activation of the reaction. Differences in the reaction conditions used here and in published studies on mesophilic ribosomes prevent direct comparison, but our data on *Thermus* ribosomes suggest that these ribosomes have adapted to changing environments using the same strategies as simple protein enzymes, balancing stability and flexibility without loss of catalytic rate. This strategy must be a very ancient process, perhaps first used by primitive ribosomes in the RNA World.**

**Keywords: ribosomes; peptidyltransferase; thermophiles; evolution; fast kinetics; puromycin**

## INTRODUCTION

Peptide bond formation is one of the most ancient enzymatically catalyzed reactions in all cells. Comparative sequence analysis of ribosomal RNAs (rRNAs) from the large subunit showed that most of the nucleotide residues in the peptidyltransferase active site are invariant (Cannone et al. 2002). More recent X-ray crystal structures have revealed a striking conservation of the three-dimensional architecture of the peptidyltransferase active site in organisms as diverse as the halophilic archaeon *Haloarcula marismortui* (Ban et al. 2000; Hansen et al. 2002, 2003), the extremely thermophilic bacterium *Thermus thermophilus* (Korostelev et al. 2006; Selmer et al. 2006), the highly radiation-resistant bacterium *Deinococcus radiodurans* (Harms et al. 2001; Schlunzen et al. 2001), and the mesophilic bacterium *Escherichia coli* (Schuwirth et al. 2005), suggesting a universal mechanism of catalysis, conserved at least since the divergence from the last universal common ancestor. However, detailed functional analyses of the peptidyltransferase reaction mechanism have been limited to ribosomes from the mesophilic species *E. coli* (Thompson et al. 2001; Katunin et al. 2002; Beringer et al. 2003, 2005; Bayfield et al. 2004; Weinger et al. 2004; Youngman et al. 2004). Just how the ribosome's catalytic activity functions in different thermal environments is unknown. To study this, we examined the kinetic and thermodynamic parameters of peptide bond formation in ribosomes isolated from the extremely thermophilic bacterium *T. thermophilus*. Here we present values for free energy of activation ($\Delta G^{\neq}$), enthalpy of activation ($\Delta H^{\neq}$), and entropy of activation ($\Delta S^{\neq}$), in an effort to better understand the adaptation of *T. thermophilus* ribosomes to an extreme thermal environment.

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## RESULTS AND DISCUSSION

### Importance of a proper A-site tRNA analog

To study the temperature rate dependence of peptidyltransferase in *T. thermophilus* ribosomes, we used f-[$^{35}$S]-Met-tRNA$^{fMet}$

 

as the P-site tRNA substrate and a saturating concentration (10 mM) of puromycin as the A-site substrate. Puromycin has been used extensively as a minimal substrate to study peptide bond formation in the ribosome (Nathans 1964; de Vries et al. 1971; Katunin et al. 2002; Beringer et al. 2003; Sievers et al. 2004; Youngman et al. 2004). At saturating puromycin concentration (10 mM), the rate constant of peptide bond formation of *T. thermophilus* ribosomes was 6 $sec^{-1}$ at 35°C and remained unchanged as the temperature was increased from 35°C to 65°C as shown in Figure 1. These results suggest that the rate between 35°C and 65°C is independent of the temperature and, therefore, is not the rate of the chemical step. We have no explanation for the lack of temperature dependence of the rate between 35°C and 65°C. The temperature sensitivity with puromycin as an A-site substrate has only been observed with a longer P-site substrate such as [$^3$H]Met[$^{14}$C]Phe-tRNA$^{Phe}$ (Sievers et al. 2004). Previous studies using f-Met-tRNA$^{fMet}$ and puromycin with *E. coli* ribosomes suggested a similar lack of temperature dependence of this reaction, the rate remaining constant between 20°C (Youngman et al. 2004) and 37°C (Katunin et al. 2002) with a $k_{cat}$ of ~1.2 $sec^{-1}$.

The rate of the peptidyltransferase reaction in *E. coli* is enhanced 10-fold when puromycin is replaced by cytidine-puromycin (CPm) as the A-site substrate (Brunelle et al. 2006; Beringer and Rodnina 2007), and the addition of the cytidine residue abolishes the strong pH dependence of catalysis observed with puromycin (Bieling et al. 2006; Brunelle et al. 2006; Wohlgemuth et al. 2006). This suggests that the cytidine moiety is important in stabilizing a more active conformation in the peptidyltransferase center by mimicking the well-described Watson–Crick interaction between C75 of the A-site tRNA and G2553 of 23S rRNA (Brunelle et al. 2006; Schroeder and Wolfenden 2007).

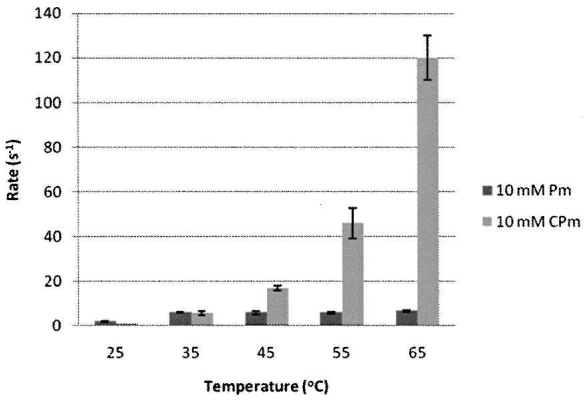


**FIGURE 1.** Temperature dependence of the rate of peptide bond formation in *T. thermophilus* ribosomes using puromycin (Pm) or C-puromycin (CPm) as an A-site substrate. The rate of the dipeptide reaction using Pm (10 mM) as an A-site substrate remains constant between 35°C and 65°C. The rate of dipeptide formation using CPm (10 mM) as an A-site substrate increases in a temperature-dependent manner.

Thus we switched to this alternative substrate (CPm) to establish the kinetic and thermodynamic activation parameters of the *T. thermophilus* ribosomes. First, we determined the $K_{1/2}$ using CPm and f-[$^{35}$S]-Met-tRNA$^{fMet}$ (Youngman et al. 2004; Brunelle et al. 2006). The rate of reaction was concentration-dependent and plateaued between 5 and 10 mM CPm, as shown in Figure 2. The concentration dependence was the same at all temperatures tested (data not shown), indicating that the $K_{1/2}$ of CPm binding is temperature-independent with a value of 3 ± 1 mM. The $K_{1/2}$ observed here is similar to those previously determined for *E. coli* ribosomes using the identical substrate (CPm) (Youngman et al. 2004; Brunelle et al. 2006).

## Temperature, rate, and the catalytic step in peptide bond formation

Using a saturating amount of CPm (10 mM), the rate of peptide bond formation increased with temperature in contrast to what was observed when puromycin was used as the substrate (Fig. 1). A linear Arrhenius plot of the rates between 25°C and 65°C (Fig. 3) indicates that no major conformational rearrangements in the active site are required for *T. thermophilus* ribosomes to function at higher temperatures. These results suggest that the rate constant determined here at saturating concentrations of CPm represents the catalytic step of the reaction.

The rate constant for *T. thermophilus* peptidyltransferase is 1.2 $sec^{-1}$ at 25°C (Fig. 1), 16-fold slower than that published for *E. coli* ribosomes at 25°C under identical substrate conditions (Brunelle et al. 2006). At 65°C, near the optimum growth temperature for *T. thermophilus* (72°C), the observed $k_{cat}$ is 120 $sec^{-1}$. Since *E. coli* rates were not measured at higher temperatures and our studies were limited to *T. thermophilus* ribosomes, we cannot directly compare rates at optimum growth conditions, but we note that with tRNAs in P- and A-sites, including EF-Tu, the calculated rate for peptide synthesis in *E. coli* ribosomes at the optimum growth temperature of 37°C is 160 $sec^{-1}$ (Johansson et al. 2008). Similar rate constants would suggest that the $\Delta G^{\neq}$ for the *T. thermophilus* ribosome at 65°C is similar to that of the *E. coli* ribosome at 37°C, and structural factors that increase or decrease thermophily enable the reactions in both ribosomes to proceed at similar rates at their respective optimum growth temperatures.

## Thermodynamic activation parameters of peptide bond formation

Thermodynamic activation parameters of *T. thermophilus* ribosomes are of special interest when studying the local flexibility of the active site, analyzing structure–activity relationships and the enzyme's ability to undergo efficient catalysis in different thermal environments (Lonhienne et al. 2000). Any difference in the free energy of activation

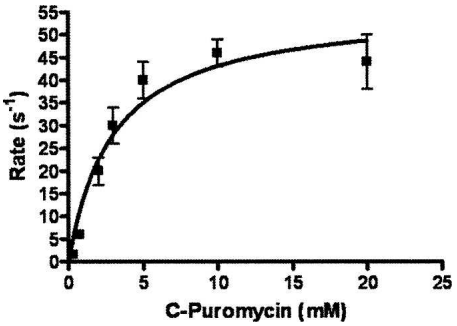


**FIGURE 2.** Concentration dependence of peptidyl transfer kinetics with cytidine-puromycin (CPm). Observed rate constants for peptide bond formation were measured by the formation of f-[$^{35}$S]-Met-CPm (dipeptide) at concentrations between 0.2 and 20 mM CPm at 55°C.

$[\Delta(\Delta G^{\neq})]$ between ribosomes from *T. thermophilus* and ribosomes from mesophiles or psychrophiles must be accompanied by changes in the enthalpy of activation ($\Delta H^{\neq}$) and/or the entropy of activation ($\Delta S^{\neq}$) as defined by the Gibbs-Helmholtz equation ($\Delta G^{\neq} = \Delta H^{\neq} - T\,\Delta S^{\neq}$). To enable future comparative studies, we have determined the thermodynamic activation parameters for peptidyl transfer by *T. thermophilus* ribosomes. The thermodynamic activation parameters as determined from the Arrhenius plot in Figure 3 are $\Delta G^{\neq}$ = 17.4 kcal/mol, $\Delta H^{\neq}$ = 22.4 kcal/mol, and $T\Delta S^{\neq}$ = 4.8 kcal/mol (25°C) for the first-order reaction by *T. thermophilus* ribosomes (Table 1).

## Conservation of the rate of peptide bond formation

It is unclear why both *E. coli* and *T. thermophilus* ribosomes have retained this remarkably fast rate for peptide bond formation. The rates of catalysis for *T. thermophilus* and *E. coli* ribosomes at their optimum growth temperatures are about 10–15 times faster than the actual overall rate of protein synthesis in the cell. Also, why is the structure so highly conserved when there is such resiliency in the peptidyltransferase region to changes introduced at key residues (Thompson et al. 2001; Katunin et al. 2002; Youngman et al. 2004; Brunelle et al. 2006)? Mutations retard the rate of peptide bond formation per se, but they do not become rate limiting for translation. Rather, other functions in protein synthesis are affected, such as decoding (Thompson et al. 2002), translocation (Fredrick and Noller 2003), and termination (Youngman et al. 2004), which also utilize these conserved residues. This may contribute to the selection against mutations and hence preserve the rate and the sequence of the peptidyltransferase region.

## Structural stability and activity in the *T. thermophilus* ribosome

The reduced activity of thermophilic ribosomes at moderate temperatures is a trade-off for acquiring a more stable structure to support the native conformation and its ability to catalyze the peptidyltransferase reaction at higher temperatures. Since a reduction of the catalytic rate at lower temperature is reflected in an increase in $\Delta G^{\neq}$ and vice versa, differences in the free energy of activation between *T. thermophilus* ribosomes and ribosomes of mesophiles or psychrophiles [defined as $\Delta(\Delta G^{\neq})_{\text{t-m/p}}$] would reflect the variation in the rate of peptide bond formation ($k_{\text{cat}}$). Enthalpic ($\Delta H^{\neq}$) and entropic ($\Delta S^{\neq}$) contributions to the value of $\Delta(\Delta G^{\neq})_{\text{t-m/p}}$ can be determined from comparing the enthalpy of activation ($\Delta(\Delta H^{\neq})_{\text{t-m/p}}$) and entropy of activation [$\Delta(\Delta S^{\neq})_{\text{t-m/p}}$]. One would predict that the slow rate of catalysis observed for *T. thermophilus* ribosomes at moderate temperatures (Fig. 1) would yield a positive value for $\Delta(\Delta H^{\neq})_{\text{t-m/p}}$ when comparing *T. thermophilus* to mesophilic or psychrophilic ribosomes at 25°C. An increase in $\Delta H^{\neq}$ decreases the $k_{\text{cat}}$ and suggests an increase in the number or strength of the enthalpy-driven rearrangements and interactions in the active site as the substrates go from the ground state to the activated state, as expected of a more thermal stable structure. Additionally, increased values of $\Delta S^{\neq}$ (more positive values) are consistent with the ground state being more ordered than the activated state. Translational, rotational, and vibrational degrees of freedom are liberated in going from the ground state to the transition state, which, in turn, increase the rate of the reaction. A decrease in $k_{\text{cat}}$ based on a positive value of $\Delta(\Delta H^{\neq})_{\text{t-m/p}}$ alone would be expected to have a dramatic effect on the rate of the reaction if there were no compensating contribution of $\Delta S^{\neq}$ to the $\Delta(\Delta G^{\neq})_{\text{t-m/p}}$. Thus we predict that variations in the $\Delta H^{\neq}$ of ribosomes due to structural changes during adaptation to different thermal environments are compensated by variations in the $\Delta S^{\neq}$. The values for the thermophilic activation parameters estimated here are shown in Table 1 along with those determined for the first-order reaction with the Phe-tRNA$^{\text{Phe}}$-containing ternary complex

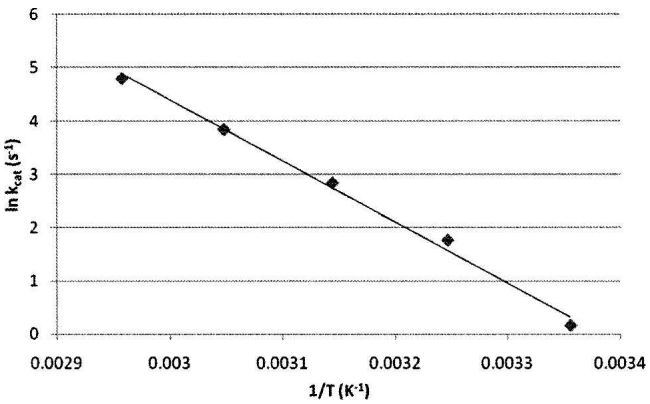


**FIGURE 3.** Temperature dependence of the first-order rate constant of *T. thermophilus* ribosome-catalyzed peptide bond formation (Arrhenius plot). The energy of activation, $E_a$, was calculated from the slope of the Arrhenius plot as described in Materials and Methods.

**TABLE 1.** Activation parameters for peptide bond formation at 25°C on thermophilic ribosomes and mesophilic ribosomes

| Ribosome | $\Delta G^{\neq}$ (kcal/mol) | $\Delta H^{\neq}$ (kcal/mol) | $T\,\Delta S^{\neq}$ (kcal/mol) |
|---|---|---|---|
| Thermophilic | 17.4 | 22.2 | 4.8 |
| Mesophilic[a] | 15.08[a] | 17[a] | 2[a] |
| Mesophilic[b] | 16.5[b] | 17.2[b] | 0.7[b] |

[a]Data from Johansson et al. (2008) (ternary complex reaction).
[b]Data from Sievers et al. (2004) (puromycin reaction).

(Johansson et al. 2008) and for the first-order reaction with puromycin (Sievers et al. 2004).

The enthalpic and entropic activation parameters reported here for a thermophilic ribosome are consistent with the entropic contribution to the rate enhancement of peptide bond formation proposed for the mesophilic ribosome (Sievers et al. 2004; Schroeder and Wolfenden 2007). Although it is difficult to accurately interpret the absolute value of the activation entropy due to the complexity of the system, the thermal stability of the thermophilic ribosome probably contributes greatly to the increase in this parameter. Increased stability of the active site would contribute to an increased order in the positioning of the substrates, thereby enhancing the rate of peptide bond formation and compensating for a decreased contribution by the activation enthalpy.

### Thermophilic as well as mesophilic ribosomes act as ''entropy traps''

Previous studies of thermodynamic activation parameters of uncatalyzed and catalyzed reactions by mesophilic ribosomes led to the proposal that the rate enhancement is entirely entropic in origin (Sievers et al. 2004). This was supported by the observed increase in $T\,\Delta S^{\neq}$ of 18 kcal/mol when compared to the entropically unfavorable solution reaction. Additionally, the enthalpy of activation within the ribosome was shown to be higher than the reaction in solution by ~8 kcal/mol. This increase in the enthalpic barrier to the reaction within the ribosome would not be expected if the ribosome acted as a chemical catalyst. The thermodynamic activation parameters for peptide bond formation in the thermophilic ribosome, calculated here, also show a high activation enthalpy accompanied by a positive activation entropy and are consistent with the proposal that ribosomes act as ''entropy traps'' by a combination of propinquity and desolvation effects (Sievers et al. 2004; Schroeder and Wolfenden 2007).

The strategy of thermal adaptation discussed here for ribosomes has also been described in homologous single-protein enzymes isolated from psychrophilic, mesophilic, and thermophilic organisms. There has been considerable focus on how protein enzymes attained optimal activity at temperatures close to their natural habitats (Lonhienne et al. 2000; D'Amico et al. 2003; Feller 2007) by a continuum of structural adaptations (Russell 2000; Vieille and Zeikus 2001; Gianese et al. 2002). The flexibility of two enzyme variants adapted to different thermal environments is very similar at temperatures near their activity optima (Závodszky et al. 1998). The data in our study show that the stability–activity relationship found in protein enzymes, as an adaptation to a wide range of thermal environments, can also be applied to a complex ribonucleoprotein such as the ribosome. Given the primordial nature of RNA-catalyzed protein synthesis, it is reasonable to speculate that this strategy of adaptation may have had its origin with the ribosome, predating its appearance in protein enzymes.

## MATERIALS AND METHODS

### *Thermus thermophilus* strain, media, and growth conditions

*T. thermophilus* strain IB-21 (ATCC 43,615) was grown aerobically at 72°C in ATCC medium 1598 (Thermus Enhanced Medium, TEM). Plates contained TEM solidified with 2.8% Difco agar.

### Ribosome purification

70S ribosomes from *T. thermophilus* IB-21 were purified as described previously (Thompson and Dahlberg 2004).

### Pepidyltransferase assays

*T. thermophilus* IB-21 70S ribosomes (1 μM) were incubated with f-[$^{35}$S]-Met-tRNA$^{fMet}$ (0.8 μM), and mini-mRNA (5′-UUUAU GUAA) (2.5 μM) in buffer A (10 mM HEPES•KOH at pH 7.6, 10 mM $MgCl_2$, 50 mM $NH_4Cl$, 1 mM DTT). Initiation factors (IFs) were not added since they had no effect on the rate or amplitude of the reaction (data not shown) and were not necessary for complex formation. Quench-flow assays were performed in a QFR-3 Kintek apparatus at temperatures between 25°C and 65°C, mixing equal 15-μL volumes of ribosome complexes and puromycin (or cytidine-puromycin), each in buffer A at the specified concentrations. Puromycin was purchased from Sigma, and cytidine-puromycin was synthesized by TriLink Biotechnologies Inc.. The reactions were quenched with 60 mM EDTA and spotted on TLC-cellulose plates for electrophoresis in pyridine acetate buffer (pH 2.8) at 800 V for 1 h (Youngman et al. 2004; Brunelle et al. 2006). Reactants and products were quantitated by phosphorimaging. Rates of the first-order reactions and dissociation constant of the substrate were determined by fitting the results with GraphPad Prism software.

### Thermodynamic activation parameters determination

The activation energy ($E_a$) was calculated from the slope of the Arrhenius plot, $E_a = -(\text{slope}) \times R$ and the enthalpy of activation from $\Delta H^{\neq} = E_a - RT$, where $R$ is the gas constant and $T$ the absolute temperature. The free energy of activation at 25°C was calculated from $\Delta G^{\neq} = RT[\ln(k_B/h) - \ln(k_{cat}/T)]$, where $k_B$ is the Boltzmann constant, $h$ the Planck constant, ($k_B/h = 2.08358 \times 10^{10}$

$K^{-1}$ $sec^{-1}$), and $k_{cat}$ is the experimental rate constant of the first-order reaction. $T\Delta S^{\neq}$ was obtained from $T\Delta S^{\neq} = \Delta G^{\neq} - \Delta H^{\neq}$. All values were expressed in kilocalories per mole (kcal/mol).

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